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Fine characterization of mitral valve glycosaminoglycans and their modification with degenerative disease

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Abstract

Background: The levels and fine structure of complex polysaccharides, glycosaminoglycans (GAGs), were determined in segments of the posterior mitral valve leaflet (MVL) taken from 15 patients affected by mitral regurgitation and degenerative disease and were compared with segments from 15 multiorgan donors.
Methods: MVL GAGs were analyzed by agarose gel electrophoresis, and by HPLC and fluorophore-assisted carbohydrate electrophoresis to evaluate disaccharide patterns after treatment with chondroitinase ABC.
Results: GAGs from the control group were composed of approximately 37% hyaluronic acid and 63% chondroitin sulfate/dermatan sulfate with a charge density of approximately 0.61. Chondroitin sulfate/dermatan sulfate polymers contained approximately 23% of the disaccharide sulfated in position 6 on N-acetyl-galactosamine, ~38% of the 4-sulfated disaccharide and ~2% of the non-sulfated disaccharide (with a 4-sulfated/6-sulfated ratio of 1.7). The total amount of GAGs was 0.66 μg/mg tissue. The total amount of GAGs in patients suffering from mitral regurgitation and degenerative disease was approximately 51.5% higher (although the difference was not significant, probably because of the low number of subjects enrolled in the study). However, significantly higher hyaluronic acid content (approx. +38%, $p<0.05$) and lower sulfated GAG content (approx. −21%, $p<0.005$) were demonstrated. As a consequence, the total charge density decreased by approximately 23% ($p<0.005$). This macromodification of GAG composition was also followed by a microalteration of the structure of the sulfated polysaccharides, in particular with a significant decrease in the 4-sulfated disaccharide (and a parallel increase in hyaluronic acid content) with no modification of the percentage of the 6-sulfated and non-sulfated disaccharides (with a significant decrease in the 4-/6-sulfated ratio).
Conclusions: We assume that changes in the relative amount and distribution of GAGs in posterior MVL in subjects suffering from mitral regurgitation and degenerative disease are consistent with a decrease in the tension to which these tissues are subjected and with an abnormal matrix microstructure capable of influencing the hydration and of conditioning the mechanical weakness of these pathological tissues.
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Keywords: chondroitin sulfate; degenerative disease; glycosaminoglycans; hyaluronic acid; mitral valve.

Introduction

The extracellular matrix (ECM) of valve leaflets consists predominantly of fibrillar collagens, elastic fibers, glycoproteins, proteoglycans (PGs) and complex polysaccharides, glycosaminoglycans (GAGs) (1, 2). The collagen, elastin and PG content of human valves has been shown to be approximately 60%, 10% and 30%, respectively, of the dry weight (1). Furthermore, the total GAGs of human heart valves have been shown to consist of mainly hyaluronic acid (HA) and chondroitin sulfates (CSs) (2, 3). In a recent study, fine characterization of normal mitral valve GAGs, in particular HA and CS/dermatan sulfate (DS), was carried out in leaflets and chordae (2) and their relative amount and distribution were found to be consistent with the tensile and compressive loads that these tissues bear. Furthermore, the relative percentage of various GAGs and their total concentration changed significantly with advancing age.

The role of the ECM and its components is not merely as a physical support; the ECM is now known to be an active, dynamic compartment capable of providing instructional signals to adjacent cells, determining many cellular functions. Thus, a wide diversity of GAGs and their parent PGs exert considerable and variable control over the physical properties of the ECM (4–7). As a consequence, the fine structure of GAGs changes during certain diseases (8, 9) that involves changes in the total amount of GAGs, such as the relative percentage of single polysaccharides, the ratio of the two CS/DS uronic acids and of 4-sulfated disaccharides to 6- and non-sulfated disaccharides of galactosaminoglycans, and the overall charge density. Therefore, CS/DS analysis, which may identify structural variations due to differential expression of the corresponding PGs and/or of the enzymes responsible for their biosynthesis, may provide evidence of tissue and cell status.

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Mitral valve repair is the preferred procedure to correct mitral regurgitation, particularly in patients with degenerative diseases with segmental prolapse of the posterior leaflet (10). The most common repair technique is quadrangular resection with simple suture plication annuloplasty (11), a highly reproducible and durable surgical operation (12). Segments of the posterior mitral valve leaflet (MVL) from 15 consecutive patients who underwent mitral valve reconstruction were analyzed for GAG percentage as well as for their fine structure in comparison with 15 multiorgan donors, with the aim of evaluating possible modifications in the content and composition of polysaccharides associated with mitral regurgitation and degenerative disease.

Materials and methods

Materials

Heparin with a molecular mass of $M_r \approx 13,100$, a charge density of 2.5, and containing approximately 40% slow-moving and 60% fast-moving moieties (13) was purified from beef intestinal mucosa. HS with a molecular mass of $M_r \approx 13,950$ and a charge density of ~1.06 was prepared from beef spleen. DS purified from beef mucosa had a molecular mass of $M_r \approx 28,200$ and a charge density of 1.09. Bovine trachea CS with a molecular mass of $M_r \approx 23,760$ and a charge density of 0.93 was obtained from the Institut Biochimique SA (Lugano, Switzerland). HA from rooster comb was from Sigma (St. Louis, MO, USA). The slow- and fast-moving components of heparin were purified as their barium salts at different temperatures, as previously reported (13). Proteinase K from *Tritirachium album* (E.C. 3.4.21.64), >500 U/mL, and chondroitinase ABC (chondroitin ABC lyase) from *Proteus vulgaris* (EC 4.2.2.4) with a specific activity of 0.5–2 U/mg, were from Sigma. Unsaturated CS/DS disaccharides were from Seikagaku (Tokyo, Japan). Stains-All (1-ethyl-2-[3-(1-ethylnaphtho[1,2-d]thiazolin-2-ylidene)-2-methylpropenyl] naphtho[1,2-d]thiazolium bromide, 3,3′-diethyl-9-methyl-4,5,4′,5′-dibenzothiacarbocyanine bromide) was from Sigma. Microcon YM-3 filters with a molecular mass cutoff of 3000 Da were from Millipore (Milan, Italy). Spectrapore dialysis tubing (M_r cutoff of 1000 Da) was from Spectrum (Rancho Dominguez, CA, USA). All other reagents were of analytical grade.

The carbazole assay for uronic acids was performed according to Cesaretti et al. (14).

Human subjects

We analyzed segments of the posterior MVL taken from 15 consecutive patients (Group B) who underwent mitral valve reconstruction, whereas only the posterior leaflet was studied in 15 multiorgan donors (Group A). Normal mitral leaflets from Group A were harvested during multiorgan explantation (cold ischemia time of 4–12 h, mean 6±2 h) and were obtained via the Cardiovascular Tissue Bank of Milan-Centro Cardiologico Monzino, IRCCS.

A total of 15 ''pathological'' MVLs (Group B) were harvested during surgical mitral repair (mean cold ischemia time 1±0.5 h). The mean age of Group B subjects was 62.3±13.5 years (9/15 patients male, 60%) and 45.46±10.6 years in Group A (6/15 subjects male, 40%). Mitral regurgitation was the principal pathology in all patients, with degenerative disease being the cause in all cases. None of these patients had associated procedures. Furthermore, we selected patients with normal echocardiographic parameters, as the control group (heart donors) comprised patients with normal hearts. Surgical patients were scheduled for mitral valve repair.

The mitral valve was approached via standard left atriotomy and cardiopulmonary bypass was established through bicaval venous return. Recognized techniques for standard myocardial protection were used, with moderate systemic hypothermia at 28°C. Once the mitral valve was exposed, intraoperative evaluation was undertaken and valve function was assessed. The repair technique used in all patients was posterior leaflet quadrangular resection and annuloplasty. The prolapsing portion of the posterior leaflet was resected and intraoperative echocardiography was used to monitor the results of repair in all patients. Samples were immediately placed dry into tubes with liquid nitrogen and, after cooling (approx. 1–5 min), were stored at −80°C until use.

Research protocols were approved by the Centro Cardiologico Monzino Medical Scientific and Ethics Advisory Committees. All mitral samples were harvested after informed consent was obtained from the patients (pathological) or the donors' relatives (normal).

Extraction of GAGs

Extraction and purification of mitral valve GAGs was performed as previously described (15–17). Briefly, approximately 50–100 mg of tissue was defatted with acetone. After centrifugation at 10,000×*g* for 10 min and drying at 60°C for 24 h, the pellet was reconstituted with 20 mM Tris-Cl buffer, pH 7.4 and treated with protease at 60°C for 12 h. After boiling for 10 min, centrifugation and filtration through a 0.45-μm filter, the filtrate was lyophilized. The powder was dissolved in 1 mL of distilled water by prolonged mixing. After centrifugation at 5000×*g* for 15 min, 0.2 mL of 20% trichloroacetic acid was added to the supernatant. After 2 h at 4°C, the mixture was centrifuged at 5000×*g* for 15 min, and the supernatant was recovered and lyophilized. After solubilization in 0.1 mL of bidistilled water and centrifugation at 10,000×*g* for 10 min, the supernatant was collected and further analyzed.

Agarose gel electrophoresis

GAGs were separated by agarose gel electrophoresis in barium acetate/1,2-diaminopropane, as previously reported (18–20). A Pharmacia Multiphor II electrophoretic system (Pharmacia LKB Biotechnology, Uppsala, Sweden) was used. Agarose gel was prepared at a concentration of 0.5% in 0.04 M barium acetate buffer, pH 5.8. Plates with a thickness of approximately 4–5 mm were prepared. Aliquots of 5 μL of the extracts were layered using micropipets. Electrophoresis was performed in 0.05 M 1,2-diaminopropane (buffered at pH 9.0 with acetic acid) for 240 min at 50 mA. After migration, the plate was soaked in a solution of 0.2% cetylpyridinium chloride and, after drying, was stained with freshly prepared toluidine blue (0.2% in ethanol/water/acetic acid 50:49:1) for 30 min, and destained with ethanol/water/acetic acid 50:49:1. Plates were also stained using a Stains-All procedure (25 mg in 500 mL of ethanol/water 50:50 overnight in the dark and destained with water) (20).

Quantitative analysis of GAGs was performed with a densitometer comprising a Macintosh IIsi computer interfaced to a Microtek Color Scanner (Microtek International Inc., Hsinchu, Taiwan). The plates were scanned in RGB mode and saved in grayscale. Image processing was carried out

using version 1.41 of the analysis program from Jet Propulsion Lab., NASA (Pasadena, CA, USA).

Strong anion exchange HPLC (SAX-HPLC)

The HPLC equipment used was from Jasco (Tokyo, Japan; pump model PU-1580, UV detector model UV-1570, Rheodyne injector equipped with a 20-μL loop, Jasco-Borwin software release 1.5). Aliquots of 20 μL of the extracted GAGs were treated with 25 mU of chondroitinase ABC in 100 mM sodium acetate buffer, pH 8.0 at 37°C overnight. The unsaturated disaccharides generated were separated by SAX-HPLC using a Spherisorb 5-SAX column (150×4.6 mm, 5 μm, trimethylammoniopropyl groups Si-CH_2-CH_2-CH_2-$N^+(CH_3)_3$ in Cl^- form; Phase Separations Ltd., Deeside, UK) using isocratic elution with 50 mM NaCl, pH 4.00 for 5 min, and gradient elution from 50 mM NaCl, pH 4.00 at 5 min to 1.2 M NaCl, pH 4.00 at 60 min at a flow rate of 1.2 mL/min. The absorbance of the effluent was monitored at 232 nm (8).

Fluorophore-assisted carbohydrate electrophoresis (FACE)

Aliquots of 20 μL of chondroitinase ABC-treated samples were lyophilized and reconstituted with 5 μL of 0.1 M 2-aminocridone (AMAC) solution in glacial acetic acid/DMSO (3:17, v/v) and 5 μL of a freshly prepared solution of 1 M sodium cyanoborohydride in water. The mixture was centrifuged in a microfuge at 11,000×*g* for 3 min. Derivatization was performed by incubation at 45°C for 4 h. Finally, 15 μL of 50% v/v DMSO was added to the samples and aliquots were taken for FACE analysis (8, 15, 18).

Statistics

The parameters determined in patients and multiorgan donors are reported as mean and SD. Differences between groups were compared by means of the t-test, with significance accepted at $p<0.05$, and were further confirmed using a multivariate approach, GLM (general linear model) with additional post-hoc testing using SPSS software for Windows (SPSS Inc., Chicago, IL, USA).

Results

MVLs from 15 multiorgan donors were analyzed (normal subjects, Group A) for GAG amounts and composition. Agarose gel electrophoresis was used to evaluate the presence of HS, DS and/or CS (20). As illustrated in Figure 1, CS and DS were found to be the main sulfated GAGs in MVLs. The absence of HS was also confirmed by treatment with nitrous acid (21) and further agarose gel electrophoresis separation (data not shown).

Determination of the disaccharide pattern of MLV GAGs by SAX-HPLC after treatment with chondroitinase ABC (Figure 2A,B, control and ''pathological'' subjects) allowed quantitative evaluation of the main sulfated GAGs, i.e., CS and DS, and quantitation of non-sulfated unsaturated disaccharide, i.e., non-sulfated CS/DS and/or HA (8, 13, 17).

Finally, FACE analysis (Figure 3) was used to quantify HA and non-sulfated CS/DS. The results obtained are presented in Table 1. MVLs from normal subjects (Group A) were mainly composed of approximately 37% HA and 63% CS/DS, with a charge density (R) of approximately 0.61 (also considering the low percentage of non-sulfated CS/DS disaccharide; see below).

The CS/DS polymers were composed of approximately 23% of the disaccharide sulfated in position 6 on N-acetyl-galactosamine, approximately 38% of the 4-sulfated disaccharide and approximately 2% of the non-sulfated disaccharide. As a consequence, the 4-sulfated/6-sulfated ratio was 1.7. The total amount of GAGs, HA and sulfated polysaccharides, evaluated by means of uronic acid assay (14), was 0.66 μg/mg tissue (Table 1). No significant differences were found between female and male control subjects, according to the study of Grande-Allen et al. (2).

A total of 15 subjects suffering from mitral regurgitation and degenerative disease (Group B, 6 females and 9 males) were also analyzed for GAG composition. The results reported in Table 1 show a quantitative and qualitative change in polysaccharide composition. The total amount of GAGs was approximately 51.5% higher in Group B, although the difference was not significant, probably due to the low number of subjects enrolled in the study. However, significantly higher HA content (approx. +38%, $p<0.05$) and lower sulfated GAG content (approx. −21%, $p<0.005$) were observed. As a consequence, the total charge density was approximately 23% lower ($p<0.005$). This macromodification of GAG composition was also followed by a microalteration in the

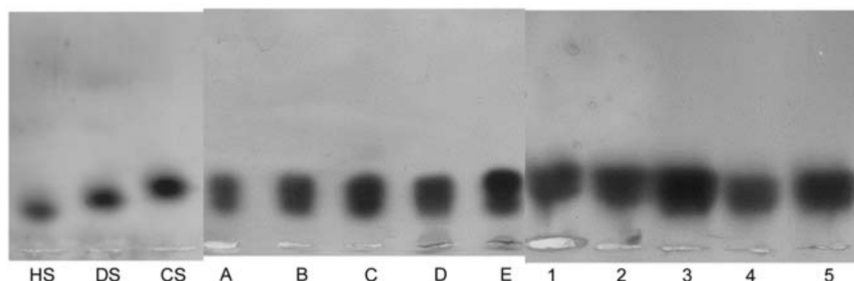

Figure 1 Agarose gel electrophoretic analysis of MVL sulfated GAGs from five (A–E) different multiorgan donors (control subjects, Group A) and from ''pathological'' subjects (Group B, 1–5) stained with toluidine blue.
HS with a molecular mass of $M_r=13,950$ and a charge density of approximately 1.06 was prepared from beef spleen. DS purified from beef mucosa has a molecular mass of $M_r\approx 28,200$ and a charge density of 1.09. CS from bovine trachea with a molecular mass of $M_r\approx 23,760$ and a charge density of 0.93 was obtained from the Institut Biochimique SA (Lugano, Switzerland). HS, heparan sulfate; DS, dermatan sulfate; CS, chondroitin sulfate.

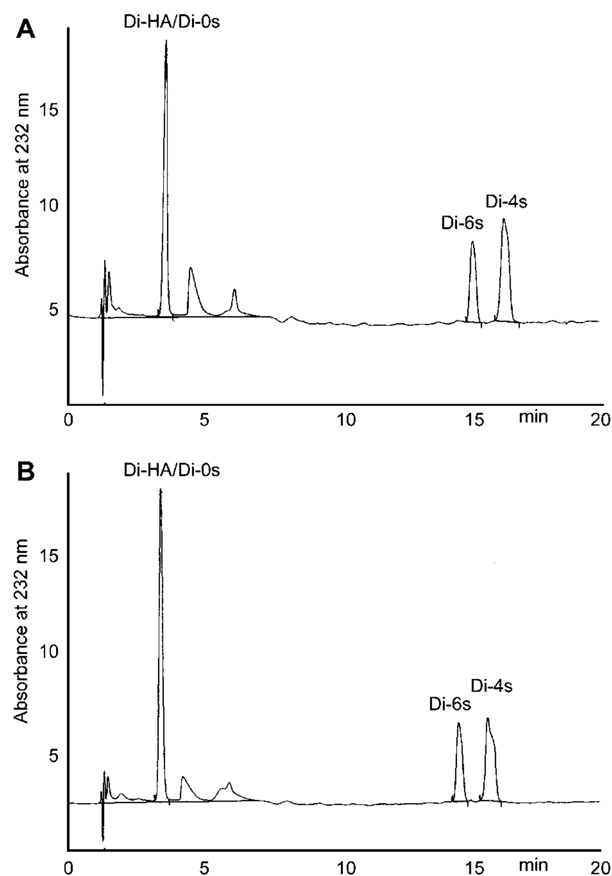

Figure 2 SAX-HPLC chromatogram of HA/CS/DS unsaturated disaccharides after chondroitinase ABC treatment of GAGs extracted from MVL of (A) control and (B) ''pathological'' subjects.

After HPLC separation, quantitative evaluation of the main sulfated GAGs, i.e., CS and DS, and of non-sulfated unsaturated disaccharides, i.e., non-sulfated CS/DS and/or HA, was determined as related unsaturated disaccharides. The percentage of each disaccharide identified was determined using purified standards (Sigma) and is reported as weight percent. Di-HA [ΔUA1→3GlcNAc], unsaturated HA disaccharide; Di-0s [ΔUA1→3GalNAc], unsaturated non-sulfated CS/DS disaccharide; Di-6s [ΔUA1→3GalNAc(6SO_4)], unsaturated 6-sulfated disaccharide (CS disaccharide); Di-4s [ΔUA1→3GalNAc(4SO_4)], unsaturated 4-sulfated disaccharide (DS disaccharide).

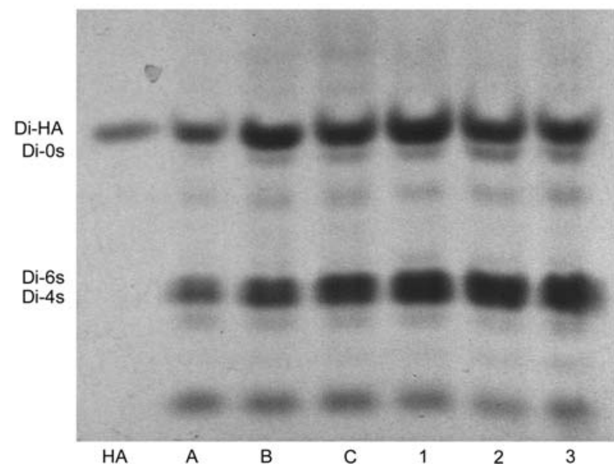

Figure 3 FACE analysis to quantify the presence of HA as the related non-sulfated unsaturated disaccharide (Di-HA) and of the non-sulfated CS/DS unsaturated disaccharide (Di-0s) of MVL GAGs from three (A–C) different multiorgan donors (control subjects, Group A) and from three (1–3) ''pathological'' subjects (Group B) after treatment with chondroitinase ABC.

The CS (Di-6s) and DS (Di-4s) unsaturated disaccharides are also illustrated. The lower bands in the gel can be attributed to the disaccharide derivatization reagents.

structure of sulfated GAGs, with significantly lower 4-sulfated disaccharide content (and a higher HA content in parallel), with no change in the percentage of the 6-sulfated and non-sulfated disaccharides. The ratio between the two sulfated disaccharides, i.e., 4- and 6-sulfated, radically changed, with a significantly lower 4-sulfated percentage (Table 1).

Discussion

In a recent paper, the fine structure of GAGs and PGs in normal MVLs and chordae was studied (2). The authors reported a different GAG composition for the anterior leaflet free edge and the posterior leaflet; in particular, approximately 50% each of HA and CS/DS in the posterior leaflet. Furthermore, approximately 80% 6-sulfated disaccharide and 20% 4-sulfated disaccharide was calculated in the posterior leaflet (2). In the present study, in the posterior leaflet of normal mitral valve we found a HA content of ~37% and CS/DS content of ~63%, with a higher percentage of 4-sulfated (38%) than the 6-sulfated disaccharide (23%), as can be observed from the example illustrated in Figure 2. This discrepancy may be mainly related to the different analytical approaches used in the studies. In particular, we directly calculated the relative percentage of non-sulfated disaccharides, i.e., HA and non-sulfated CS/DS disaccharides, and the 4-/6-sulfated disaccharides by means of HPLC after treatment with chondroitinase ABC. In contrast, Grande-Allen et al. evaluated these disaccharides by FACE analysis after treatment with hyaluronidase SD and chondroitinase ABC or chondroitinase ACII and further drying and fluorotagging of the disaccharides (2). As a consequence, the use of different analytical approaches may produce slight differences in the quantitative results. Moreover, a highly significant difference in GAG amounts and type was calculated by Grande-Allen et al., depending on the age of normal subjects from approximately 20 to 75 years (2). In contrast, our normal and patient groups were characterized by a very narrow age range, approximately 11–13 years around the mean value. As a consequence, differences in GAG quantitative values compared to the study of Grande-Allen et al. based on normal subjects of 20–75 years (2) may also be related to the different normal subject groups.

The quality and distribution of GAGs has been associated with the tensile or compressive loading of normal mitral valve regions. In fact, the regions of the valve that experience tension were found to contain less HA but more galactosaminoglycan sulfated in position 4 of N-acetyl-galactosamine [DS, considering

Table 1 Composition, amount and charge density of MVL GAGs in controls and patients suffering from mitral regurgitation and degenerative disease.

	Controls Group A	Pathological Group B	Difference, %	Significance
GAGs, μg/mg tissue	0.66±0.26	1.00±0.65	+51.5	NS
HA, %	36.6±15.5	50.4±11.9	+37.7	p<0.05
CS/DS, %	63.4±15.5	50.3±11.9	−20.7	p<0.005
Charge density	0.61±0.2	0.47±0.1	−23.0	p<0.005
Di-0s, %	2.2±1.4	2.5±1.6	+13.6	NS
Di-6s, %	22.9±5.7	21.1±6.4	−7.9	NS
Di-4s, %	38.3±12.1	26.1±6.4	−31.9	p<0.001
4s/6s	1.67±0.5	1.24±0.4	−24.0	p<0.005

Results are presented as mean±SD. Differences and significance between the groups are also reported. Charge density was calculated by considering the number of sulfated groups per disaccharide unit.

that iduronic acid-containing units are often sulfated at C4 of the N-acetyl-galactosamine residue (22)] and less water. On the contrary, valve regions associated with compression contained more HA and galactosaminoglycan mainly sulfated in position 6 of N-acetyl-galactosamine [CS, considering that sulfation at C6 is frequently associated with glucuronic acid-containing disaccharides (22)] but significantly less DS (2).

We studied the GAG composition in posterior MVLs of a normal group with a mean age of ~45 years and in a pathologic group with a mean age of ~62 years. However, this difference in mean age of the two groups is not representative of the modifications related to age because of the minor differences in mean age (17 years). In fact, significant differences were only found for age groups with a greater age range, from ~20 to 75 years (2). In the subjects suffering from mitral regurgitation and degenerative disease, we found significantly higher HA content (approx. +38%, $p<0.05$) and lower sulfated GAGs (CS/DS) and, as a consequence, a lower total GAG charge density in the posterior MVL is compatible with a decrease in tension associated with mitral regurgitation and degenerative disease to which these tissues are subjected. This is also compatible with the lower content of galactosaminoglycans sulfated in position 4 (i.e., DS) and the parallel lower 4s/6s ratio with the relatively higher content of galactosaminoglycan sulfated in position 6 of N-acetyl-galactosamine (i.e., CS). Finally, we assume that changes in the relative amount and distribution of GAGs in the posterior MVL of subjects suffering from mitral regurgitation and degenerative disease are consistent with an abnormal matrix microstructure capable of influencing the hydration and of conditioning the mechanical weakness of these pathological tissues. This is confirmed by previous studies on myxomatous mitral leaflets and chordae (23, 24), in which an increase in overall GAG concentration from 30% to 150% was measured, with significantly more 6-sulfated groups and slightly more HA. Furthermore, this hypothesis is also consistent with present knowledge regarding the remodeling capacity of the ECM due to pathological conditions (25) to provide new structural support and to modulate cellular behavior by activating signaling pathways. In fact, in relation to heart tissues, mitral valve prolapse (26), myxomatous changes (23, 24), myocardial infarction (25) and ischemia (27) were found to be associated with dynamic changes in the ECM composition in animal models and humans. In these studies, HA was considered to play a key role in cell adhesion during wound healing and in promoting cell division.

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